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Capturing Single Molecules of Immunoglobulin and Ricin with an Aptamer-Encoded Glass Nanopore

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Abstract

Nanopore-based single-molecule biosensors have been extensively studied. Protein pores that have receptors attached to them are target-selective, but their real-time applications are limited by the fragility of the lipid membrane into which the protein pores are embedded. Synthetic nanopores are more stable and provide flexible pore sizes, but the selectivity is low when detecting in the translocation mode. In spite of modifications with probing molecules, such as antibodies, to potentiate specific targeting, these nanopores fail to bind individual target molecules. Distinguishing between binding and translocation blocks remains unsolved. Here, we propose an aptamer-encoded nanopore that overcomes these challenges. Aptamers are well-known probing oligonucleotides that have high sensitivity and selectivity. In contrast to antibodies, aptamers are much smaller than their targets, rendering target blockades in the nanopore much more distinguishable. We used aptamer-encoded nanopores to detect single molecules of immunoglobulin E and the bioterrorist agent ricin, sequentially captured by the immobilized aptamer in the sensing zone of the pore. The functional nanopore also probed sequence-dependent aptamer-protein interactions. These findings will facilitate the development of a universal nanopore for multitarget detection.

INTRODUCTION

Nanopore technology has revolutionized single-molecule detection. When different target molecules traverse or bind in the nanopore, they characteristically block the ion pathway, yielding a conductance change that serves as a signature for target identification and quantification^{1,2}. This capability renders nanopore technology useful in numerous applications, including biosensing^{1–5}, nucleic acids (potential for DNA sequencing) and peptide detection^{6–18}, control of molecular transportation^{19;20}, and the study of single-molecule chemistry²¹.

The nanopores assembled by protein ion channels allow for the attachment of a receptor in the lumen through structure-directed genetic engineering and chemical modification²². The receptor recognizes the specific target, making the protein pore highly selective. The limitation of protein pore sensors is that they are embedded in a very fragile lipid bilayer. New technologies, such as robust ion channel-integrated biochips^{23;24} and the lipid bilayer-supported glass nanopore membrane²⁵, are emerging to overcome this challenge. Compared with protein nanopores, the synthetic nanopores, created on solid supports using micro- and

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nano-technologies^{12;15;26–35}, offer higher stability, flexible pore sizes, and array platforms; these greatly expand the potential of nanopore applications in biotechnology and the life sciences^{36–39}. However, the greater stability of solid nanopores comes with reduced selectivity. Single molecules, such as DNA or proteins, are measured during translocation through the pore, but any molecules smaller than the nanopore also can generate a transient pore block. This low specificity limits the ability to identify and isolate molecular processes with translocation-based detections⁴⁰. This may be overcome by coating a layer of probing molecules to the nanopore^{5;15;41–43} to force specific targeting. For example, DNA transport was enhanced in nanopores modified with an complementary oligonucleotide probe^{15;41}; a biosensing nanopore coated with antibodies was fully blocked by the target protein⁵; and recently, a significant step was made toward functionalization at selected points in a solid nanopore⁴². However, these methods have thus far failed to detect the binding of individual target molecules, and it remains impossible to distinguish between blocks produced by binding and those generated by translocation.

In this report, we describe the integration of sophisticated aptamers and the glass nanopore technique⁴⁴ that paves an avenue to overcome these challenges.

Aptamers, or “synthesized antibodies,” are short DNA or RNA segments that are created by *in vitro* evolution^{45;46}. Despite their diminutive size, aptamers recognize and specifically bind broad species of ligands, such as peptides⁴⁷, proteins^{48–50}, and pathogenic targets^{51–53}, with high affinity (nano- to picomolar)⁵⁴ that matches or exceeds that of their true antibody counterparts⁵⁵. Because aptamers are much smaller than their targets, when they are bound by the target, the target signal is pronounced, allowing one to identify single molecules that are sequentially captured by the immobilized aptamer. Therefore, aptamers outperform antibodies with regard to single-molecule detection in nanopores.

Certain properties of the glass nanopore⁵⁶ also facilitate single-molecule detection. This wineglass-shaped nanopore, fabricated on a micropipette tip with a freely manipulable pore size, can be fashioned to accommodate nearly any molecular complex and has a series of benefits: ease of fabrication by virtually any laboratory at low cost; precisely manipulated pore size, from 1 to several hundred nanometers; an experimentally verified capacity to capture single molecules and perform stochastic sensing; reduced electrical noise; biofriendly surface engineering; and the ability to perform as a probe platform for *in situ* and high-throughput applications.

We used aptamer-encoded nanopores to detect 2 important targets, immunoglobulin E (IgE) and ricin. IgE is an immunoglobulin that is found at its lowest levels in human serum and is consistently a representative biomarker for clinical detection methods. Abnormal levels of IgE are associated with allergy-mediated disorders and immune deficiency-related diseases, such as AIDS. The bioterrorist agent ricin is the third-most toxic substance, after plutonium and botulinum toxin, according to the US Environmental Protection Agency and the Center for Defense Information. Ricin has high potential for use as an agent of biological warfare, a weapon of mass destruction (WMD), and a terrorist weapon.

EXPERIMENTAL SECTION

Detailed procedures for the glass nanopore fabrication and electrical measurements have been described in a recent report⁴⁴. The nanopore was functionalized in two steps, surface silanization and aptamer attachment. To silanize the glass surface of the nanopore, the nanopore-terminated pipette was cleaned by filling with 1 M NaOH and incubating at 100 °C for 30 mins, followed by rinsing with 5% HCl for 5 mins, sonication (15 kHz) for 1 s, and washing with double deionized water (ddH₂O) for 5 times. The cleaned pipette was dried at 100 °C for 30 mins. The pipette terminal was filled with a 90% methanol/water

solution containing 2% Aldehyde methoxysilane (United Chemical Technologies, Bristol, PA) and incubated at room temperature for 2 hrs, followed by rinsing with 90% methanol more than three times, and sonication for 1 s. The silanized pipette was dried at 100 °C for 30 mins. DNA and RNA aptamers were synthesized, terminal-modified, and HPLC-purified by Integrated DNA Technologies Inc. The aptamer names, references, sequences and terminal modifications are summarized as the follows:

Anti-IgE aptamer (A_{IgE})^{57–59}, 5'-GGGGCACGTTTATCCGTCCCTCCTAGTGG
CGTGCCCC-NH₂-3' (37 bases)

Control DNA aptamer⁵⁹, 5'-
CCCCGTGCGGTGATCCTCCCTGCCTATTTGCACGGGG-NH₂-3' (37 bases)

Anti-ricin A-chain RNA aptamer (A_{ricin})^{60;61}, 5'-NH₂-C6-
GGCGAAUUCAGGGGACGUAGCAAUGACUGCC-3'

To immobilize the aptamer on nanopore surface, the silanized pipette tip was filled with 1 μL of the aptamer solution. The aptamer solution was a PBS⁺ solution (PBS solution plus 1 mM MgCl₂, pH7.4) containing 2 μM amino-terminated DNA or RNA aptamer, 2 mM sodium cyanoborohydride as a reducing agent and 15 mM NaN₃. To ensure the aptamer solution reaches the narrow end of nanopore, the pipette tip was also inserted into a pool of the same aptamer solution as inside the pipette. The glass nanopore was incubated with aptamer and sodium cyanoborohydride at room temperature for overnight, sonicated for 1 s, and rinsed with ddH₂O for three times.

In all electrical measurements, the external solution was grounded and the voltage was given from the internal solution, as is conventional for voltage polarity. The nanopore-terminated pipette was filled with a 1 M NaCl recording solution and merged into the same recording solution as was inside the pipette to measure the current at a voltage applied. The conductance was calculated from the current value. The pore size was evaluated based on the pore size-conductance correlation (1 M NaCl), as previously established⁴⁴.

Human Myeloma Plasma IgE and Human Plasma IgG were purchased from Athens Research & Technology, Athens GA. The purities of IgE and IgG were greater than 95% by SDS polyacrylamide gel electrophoresis according to the company. The ricin A-chain protein was purchased from Sigma, St Louis MO. All the targets were diluted in the PBS⁺ solution to appropriate concentrations. The divalent metal ion Mg²⁺ in the PBS⁺ solution is necessary for forming the tertiary structure of aptamers^{57;60}. The binding of targets to the aptamers in the nanopore was electrically monitored in PBS⁺ solution present in both the pipette and external bath. Data were given as the mean $\pm$ SD, based on at least three separate measurements.

RESULTS

Nanopore fabrication

To fabricate the nanopore⁴⁴, we sealed the pipette tip with a melting process so that a wineglass-shaped nanocavity was formed inside the terminal (Fig. 1a). The tip was exposed to hydrofluoric acid/ammonium fluoride for external etching, and monitored by the ionic current between solutions inside and outside the pipette (Fig. 1b). A nanopore is formed once the enclosed nanocavity was perforated (Fig. 1c). The pipette tip was then transferred to an etchant-free solution to determine the pore conductance. The tip can be repeatedly etched until the desired conductance is achieved. To be consistent with our previous report⁴⁴, the conductance for each nanopore has been determined in 1 M NaCl. Due to the uniform nanocavity profile, the corresponding pore size can be evaluated from the conductance according to the pore size-conductance correlation⁴⁴. The values of conductance and pore

sizes were given in Table S1 in Supplementary Materials. There are a number of methods of coupling terminal-modified oligonucleotides with activated glass surface⁶². For example, in an optimized method, the amino-terminated IgE-binding aptamer can be immobilized on the amino-silanized glass slide via glutaraldehyde linkage, with an enhanced coupling efficiency by using sodium cyanoborohydride (NaBH_3CN) as a reducing agent⁵⁹. We employed a similar but simplified process for immobilizing the aptamer on the nanopore: silanizing the glass surface in the pore with aldehyde methoxysilane, followed by attaching amino-terminated DNA or RNA aptamers to the aldehyde-terminated glass surface in sodium cyanoborohydride (in Methods). This reaction has been successfully applied in protein attachment to the silicon nanowire⁶³.

Capture of single IgE molecules in the aptamer-modified nanopore

We first observed that, in the absence of the target IgE, the current of a 63 nm nanopore modified with the anti-IgE aptamer (A_{IgE})^{57–59} remained flat without any discrete change (Fig. 2a). After injecting a solution of 1 μl IgE (5 nM) into the pipette terminal around the nanopore via a thin plastic tube (i.d. $\sim 100\ \mu\text{m}$), we identified a series of stepwise current blocks, as shown in Fig. 2b. There was no such blocks when IgE was presented in an unmodified nanopore, or in a pore modified with the control aptamer that is different from A_{IgE} (data not shown), suggesting that both IgE and immobilized A_{IgE} are necessary for stepwise blocks, and the possibility of current variation by non-specific adsorption is excluded. The stepwise blocks are also distinguished from those produced by protein molecule translocation, as protein molecules traversing a nanopore typically generated short-lived blocks with a duration ranging from $10^2\ \mu\text{s}$ to $10^2\ \text{ms}$ ^{36–38}. We proposed that the stepwise blocks are associated with single IgE molecules that sequentially bind to the immobilized aptamers in the nanopore, one molecule per block level. To confirm this binding process, we pre-mixed IgE (5 nM) with A_{IgE} (50 nM). When the mixture was added in the A_{IgE} -modified pore, no stepwise blocks were produced. We reasoned that, due to the high affinity of $\text{IgE} \cdot A_{\text{IgE}}$ ($K_d \sim 10\ \text{nM}$)⁵⁷, most IgE molecules were already bound with A_{IgE} in the pre-mixed solution before it was presented to the pore; therefore, little free IgE was left to bind with the immobilized aptamer.

By counting the stepwise blocks, we determined the number of observed IgE molecules that bound. The analysis indicated that there were, at most, ~ 20 IgE blocks in a nanopore (Fig. 2b). This number was compared with that of immobilized aptamers. The nanopore can be simply treated as a cone-shaped pore with a narrow opening of 60 nm (based on average pore conductance) and an aperture of 60° (based on nanopore profile)⁴⁴. The 1 μl of aptamer solution (2 μM) loaded in the pipette terminal would interact with $\sim 4\ \text{nm}^2$ of the inner surface. According to the reported optimum immobilization density of $5\ \text{nm}^2$ per DNA molecule on glass⁶⁴, this area allows for attachment of $\sim 10^{11}$ aptamer molecules. We did not find from literatures the efficiency for our immobilization method, but the attached aptamer should be more than bound IgE molecules by at least several orders of magnitude. The finite number of IgE blocks can be interpreted as the sensing zone of the nanopore (Fig. 2c). We proposed a sensing zone similar to that identified in conical nanopores in the track-etched polymeric film^{38;65;66}. That sensing zone was localized at the narrow opening of the pore, and was highly responsive to molecular translocations due to higher intensity of electrical field than the other regions. In our nanopore, although a large number of aptamers were immobilized, only single IgE molecules captured by aptamers in the sensing zone generated pronounced signal blocks, whereas molecules outside of the sensing zone yielded signal blocks that are too weak to be detected. Based on the wineglass-shaped profile, we approximated the thickness of sensing zone. Assuming the effective diameter of IgE (190 kDa) is 11 nm, the same as that of IgG (150 kDa)⁶⁷, one IgE molecule should occupy $\sim 100\ \text{nm}^2$. Thus 20 IgE molecules should cover at least $2,000\ \text{nm}^2$ of nanopore surface. For a pore

with the geometry described above (60 nm in opening, 60° in aperture), the IgE-covered area would fall into a at least 9-nm-thin sensing zone beginning at the pore opening. This thickness is comparable to the IgE dimension (11 nm), suggesting that the sensing zone comprises a single-molecule ring of IgE (Fig. 2c).

The drop in conductance of these blocks (Δg) ranged from 0.16 to 2.1 nS, representing an average decrease of 0.4% in nanopore baseline conductance (Fig. 2b). The large variation of Δg was also observed for the ricin blocks, which was shown in Fig. 5a. This phenomenon could be related to the binding location in the pore, as a more effective current block by the analyte occurs in the narrow opening than farther from the tip^{38;56}. A recent simulation study also identified a pore width-dependent current component that lines the charged pore surface⁶⁸, which is larger at the narrow opening than it is deeper inside the pore. Because the larger area that is deeper in the pore allows for binding more molecules than the narrow opening, there will be more small Δg blocks than large Δg blocks. This expectation was verified, based on the distribution of Δg in the histogram (Fig. 2d). In addition, the asymmetrical target molecules that bind in different orientations can also affect the drop in conductance. To this end, we have found that single symmetrical gold nanoparticles (10 nm) that bind in the pore can produce stepwise current jumps with a smaller variation of Δg (unpublished data).

In contrast to the number of stepwise single-molecule blocks, we rarely observed characteristic current change for releasing IgE from immobilized aptamers. For example, no discrete current increase was identified in the 40-minute recording shown in Fig. 2b, suggesting a long binding duration for IgE•A_{IgE} in hours, which is equivalent to a dissociation rate constant (k_{off}) of $\sim 10^{-5} \text{ s}^{-1}$. The slow dissociation rate for IgE•A_{IgE} has been previously described, with different methods of aptamer immobilization and materials of solid support: k_{off} for IgE binding to the amino-terminated aptamer was $3 \times 10^3 \text{ s}^{-1}$; and to the biotinylated aptamer was $5 \times 10^{-4} \text{ s}^{-1}$, with both aptamers attached to gold surfaces⁵⁸. By comparison, the dissociation rate in our glass nanopore was slower, suggesting that the confinement in the nanopore could enhance the bonding strength between the target and its aptamer. To determine the association rate constant, we analyzed the detection time to bind each IgE (t). t measures the duration from the start of the current recording to the occurrence of an IgE block (Fig. 2e). The current recording was started immediately after the protein was delivered to the inner terminal around the nanopore. The gap between the protein injection and current recording was less than 5 seconds and was negligible compared with the much longer detection time. The relation of t and the number of block n was fitted exponentially. The time constant τ_{on} was $18 \pm 5 \text{ mins}$ ($n=6$). Using τ_{on} , the apparent association rate, k_{on} , was calculated as $1.9 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ ($k_{on}=1/(\tau_{on} \cdot [\text{IgE}])$). This association rate constant is similar to $3 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ calculated based on the previously reported K_d (10 nM)⁵⁷ and k_{off} ($3 \times 10^{-3} \text{ s}^{-1}$)⁵⁸. We also verified the electroosmotic flow based on the voltage polarity-dependent blocks, i.e. no stepwise block was observed at -100 mV (data not shown), as it was at $+100 \text{ mV}$. The voltage polarity switches the direction of the electroosmotic flow, thus enhancing or weakening the binding frequency⁴⁴. The electroosmotic force can accelerate the transit of proteins to nanopore, therefore it is unlikely that all of the protein in the pipette bound to the immobilized aptamers before reaching the nanopore.

Selectivity of the aptamer-encoded nanopore

To assess the potential for use in biosensing applications, we investigated the selectivity of the aptamer-encoded nanopore. We first compared the response of A_{IgE}-modified nanopore in different molecular species. Fig. 3a shows the current trace when 5 nM IgG was presented in a 53 nm A_{IgE}-modified pore ($+100 \text{ mV}$). Although IgG is similar to IgE in molecular dimension, the addition of IgG did not give rise to stepwise blocks as IgE did (Fig. 2b),

indicating that no IgG was captured. After testing with IgG, we continued to add IgE in the same pipette, and found similar stepwise blocks as in Fig. 2b (data not shown), confirming the specific binding of IgE to the aptamer. Further, we studied the affinity of IgE for the mutant aptamer, C20. In this aptamer variant, a single nucleotide, cytosine 20, has been deleted from the center of the hairpin loop. The current trace in Fig. 3b shows that the presence of IgE in a 57 nm Δ C20-modified pore (460 nS) did not yield stepwise blocks as in the A_{IgE}-modified nanopore (Fig. 2b), but did generate short single-leveled blocks, which lasted 0.68 ± 0.31 mins ($n=3$). The block starts with the binding of IgE, and ends with the release of IgE from immobilized Δ C20. Clearly, the removal of a single nucleotide from the aptamer dramatically reduces the binding duration, indicating the critical role of the hairpin sequence of A_{IgE} in the interaction with its target⁵⁷.

Detection of IgE in the exterior solution with nanopore

The aptamer-encoded nanopore can also detect single molecules in the exterior solution. This is a useful detection mode for real-time applications. In this detection, we began recording the current immediately after merging the functional nanopore into the solution that contained the target protein. Different from IgE in the interior solution, a negative voltage should be applied to allow external IgE to enter the pore, driven by the electroosmotic flow. Fig. 4a shows the model and Fig. 4b the current trace for a 55 nm pore (-100 mV) in the presence of 5 nM external IgE. The stepwise blocks are similar to those by IgE added in the pipette (Fig. 2b), but all the blocks were in the upward direction because of the negative current. We attributed the stepwise blocks to the binding of external IgE to the immobilized aptamer in the pore and eliminated the possibility that the protein bound to the exterior surface of pipette, on which no aptamer was immobilized. After the external application of IgE, we filled in the pipette with the same IgE solution. No additional blocks were detected (Fig. 4c), indicating the IgE from the external solution saturated the binding sites.

The protein in the exterior bath can also be quantified. Suppose that the detection time from each block t_n follows the exponential distribution $n = n(1 - e^{-t_n/\tau})$. The constant τ is inversely proportional to the target concentration—i.e., $\tau = 1/(k_{on} \cdot [\text{Target}])$, where k_{on} is the associate rate constant. The target concentration can be obtained from τ . In a low target concentration (nM to pM), however, the detection time will be prolonged (minutes to hours) due to the fewer events that can be detected in the same duration. We therefore used the interval between the 1st and 2nd blocks ($\Delta t_{12} = t_2 - t_1$) as a meaningful parameter. Δt_{12} is

expressed as $\Delta t_{12} = t_2 - t_1 = \tau \ln(\frac{n_0 - 1}{n_0 - 2}) = C\tau$, which indicates a linear correlation between Δt_{12} and τ —and thus the target concentration—supporting the validity of Δt_{12} in correlation with the target concentration. The use of Δt_{12} instead of t_1 or t_2 also resolves the issue of when to start timing. Fig. 4d shows the variation of Δt_{12} for a broad range of IgE concentrations, in which Δt_{12} decreased from 130 mins to 3.7 mins as the concentration of IgE increased from 500 fM to 5 nM.

Detection of single ricin molecules with the RNA aptamer-encoded nanopore

Because the nanopore can be potentially functionalized with any aptamer, our aptamer-encoded nanopore should be programmable. Besides DNA, we can also attach RNA aptamers that target high impact substances to the pore. Ricin is such an important example. Ricin is a protein toxin from the castor bean plant that has high potential as a bioweapon agent. Ricin protein consists of the A chain and B chain. The A chain has the toxic enzymic activity (an RNA N-glycosidase) of the protein and the B chain responsible for binding to cells and being taken up by them. The ricin A protein has 267 amino acids with a molecular weight of 34 kD, and adopts a highly ordered conformation that has a long axis of ~ 6.1 nm

and a short axis of ~ 2.4 nm⁶⁹. The toxicity of ricin is activated only when the A chain meets with B chain. The RNA aptamer targeting ricin A protein has been created by Ellington and co-workers⁶⁰. recently the ricin A aptamer has been successfully integrated into the chip for ricin detection⁶¹. However, the detection requires fluorescent-labeling of the ricin protein. Here, we have demonstrated the novel, label-free capture of single ricin molecules using an A_{ricin}-encoded nanopore. The current characteristics of the A_{ricin}-modified nanopore (56 nm, -100 mV) applied to a solution containing 100 nM ricin A-chain protein (Fig. 5a) resemble that of the A_{IgE}-modified pore in the IgE solution (Fig. 2b and Fig. 4a). First, a series of stepwise blocks were observed; these were attributed to the capture of single ricin molecules. The average detection time for these blocks was 10 ± 5.2 mins (n=3); further, no discrete current increase was observed for the release of ricin from its aptamer, suggesting a long binding time for A_{ricin}•ricin. This property is similar to the binding time for A_{IgE}•IgE, and is also in concordance with the high affinity (7.4 nM) for A_{ricin}•ricin reported earlier⁶⁰. We used a similar method as the IgE assay to measure the concentration of ricin in the exterior solution. For each detection, we determined the interval between the first and second block, $\Delta t_{12}=t_2-t_1$, and correlated Δt_{12} with the ricin concentration in Fig. 5b. Δt_{12} decreased from 35 mins to 2.6 mins as the ricin concentration increased from 2.8 nM to 2.8 μ M.

DISCUSSION

The integration of sensitive aptamers with the glass nanopore-terminated probe may overcome the main challenges encountered in most nanopore-based single-molecule biosensors.

First, this nanopore adopts the receptor attachment mode, rather than the molecular translocation mode, for single-molecule detection. Only molecules that are recognized by the immobilized receptor are able to yield featured block signals, giving rise to the nanopore selectivity. Although translocation events also occur, they can be easily recognized from their duration. For example, the duration of IgE translocation in the trace shown in Fig. S1 was 6.7 ± 3.1 ms, a time scale that is comparable with other nanopores^{38;56}. Because the translocation duration is several orders of magnitude shorter than the binding events ($\sim 10^2$ min), the translocation event can be simply excluded when analyzing long binding blocks. The translocation frequency is related to the pore size and target species. Fig. 2b for IgE shows fewer translocation events than Fig. 5a for ricin. The average frequency of 2.1 min^{-1} (5 nM IgE, +100 mV) is slightly higher than the 0.5 min^{-1} for stepwise binding blocks in the same trace and is in the same order of magnitude as protein translocation in other nanopores³⁸.

Second, by using aptamers as the receptor, our nanopore gains the ability to produce stepwise single-molecule blocks, which has not been reported in other functionalized nanopores. Unlike antibodies, aptamers are much smaller than their targets, making target blockades much more distinguishable. Antibodies have been attached in polymer nanopores for protein detection⁵, but single-molecule binding events were not observed; the pore conductance had to be fully blocked by molecules in order for protein quantification, requiring a pore size that matched the target molecular dimension. By comparing, through stepwise blocks, we were able to “visualize” single protein molecules that were sequentially captured in the aptamer-encoded nanopore. In addition to aptamers, the low electric noise in the glass nanopore also facilitates the separation of single-molecule binding events. Achieving low noise is still a challenge for many nanopore systems. The discrete single-molecule blocks are particularly useful in real-time applications where the background current drifts or fluctuates along over time, because the digital signal of the discrete blocks distinguishes them from the analog background signal. Moreover, with aptamer-encoded

nanopores, the pore size does not need to match the target molecule dimension. Of all the nanopores tested, we have observed the stepwise blocks for IgE and ricin A chain with a broad range of pore size from ~40 nm to ~100 nm. Since the detection is less dependent on the pore size, one size pore may be suitable for the detection of different sized molecules without losing specificity.

Third, aptamer-encoded nanopores can simultaneously detect multiple long binding events with multiple aptamers as probes at single-molecule resolution. Such interactions that are governed by slow dissociation dominate many significant bioprocesses, such as high-potency inhibitors (including aptamers) for enzymes. This nanopore allows multiple aptamers to be constructed as probes in a single pore, each aptamer capturing a molecule. By observing multiple molecules binding to their probes, many long binding reactions can be detected at the single-molecule level. To detect binding that has a long duration, such as target•aptamer reactions, the multiprobe detection mode is more sensitive than single-probe detection, because the frequency of binding occurrences (s^{-1}) should increase with probe number. It has been reported that the sensitivity of ion channel-based stochastic sensing increases linearly with the number of channels in the lipid bilayer, provided that each channel functions independently⁷⁰. Technically, constructing multiple probing aptamers is also easier than building only 1 probe in the nanopore.

Currently, the long sensing time at low concentration is an issue that applies to our and other nanopores. It takes $10^1 - 10^2$ minutes to detect a target in the pM range using aptamer-encoded nanopore; 200 minutes is spent for 1 pM protein analyte to completely clog an antibody-coated nanopore for analyte quantification⁵; To sense drug compounds using the α -hemolysin protein pore, the block frequency was $250 s^{-1}$ in $160 \mu M^3$, which is equivalent to $0.1 min^{-1}$ in 1 nM, or 10 minutes to detect 1 binding event. How can binding frequency increase at very low concentrations so that the sensing time can be shortened? This issue is related to the diffusion-controlled collision rate and activation energy for binding. The frequency of binding events could be effectively increased by convective transport: the transport of charged molecules, such as DNA, can be accelerated electrophoretically, and neutral molecules, such as IgE and ricin, can do so by electroosmotic flow. In general, convective transport can enhance the binding opportunity in the nanopore. This problem can also be improved by using a nanopore array that is fabricated from the capillary array, which multiplies the binding frequency, enhancing the sensitivity. In addition, the preconcentration that is used in many trace analysis systems can increase the amount of trace constituents (IUPAC Compendium of Chemical Terminology, 2nd Edition (1997)).

Although nonspecific clogging can also occur in detecting targets in real complex samples, we have never observed it with proteins in the pipette or external bath. Clogs by charged particles, such as DNA, can be removed by applying high voltages, and the likelihood of clogging by large molecules can be reduced by prefiltering the samples.

In conclusion, we have constructed an aptamer-encoded nanopore that allows for selective and sensitive protein detection at single-molecule resolution. We will continue to improve the aptamer-encoded nanopore to develop a real-time sensor. In the future, a functional nanopore with an aptamer can be a tool for exploring various bioprocesses involved in bioanalysis⁷¹⁻⁷⁶, diagnostics⁷⁷, therapy⁷⁸, biocatalysis, and cell modulation⁷⁹. Aptamers are also suitable for nanopore-based read-time detection, because aptamers are more durable than most protein receptors, resisting most denaturing and degrading conditions, including immobilization⁷⁴; yet, they are simpler to synthesize, modify, and immobilize using low-cost methods. The affinity and specificity of aptamer-target interactions can also be fine-tuned through rational design or molecular evolution. These properties will benefit an advanced strategy for multitarget detection with a universal nanopore.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Fabrication of the nanopore. **a.** Microscopic image of a sealed micropipette tip enclosing the nanocavity. **b.** Cartoon depicting the external etching of the pipette tip. **c.** Perforation of nanocavity to form a nanopore.

Figure 2.

Capture of single IgE molecules by immobilized aptamers in the nanopore. The voltage was given from the electrode in the pipette and the external bath was grounded. **a.** Current in a 63 nm A_{IgE} -encoded nanopore at +100 mV without IgE in the pipette. **b.** Current through the same pore as in a, with 5 nM IgE in the pipette. **c.** Sensing zone at the narrow opening of the nanopore. **d.** The detection time of each sequentially-occurring stepwise block in b. **e.** Amplitudes of conductance change for stepwise blocks.

Figure 3. Selectivity of aptamer-encoded nanopores. **a.** Current for 5 nM IgG in an 53 nm A_{IgE}-modified nanopore at +100 mV. **b.** Current for 5 nM IgE in a 57 nm Δ C20 (mutant of A_{IgE})-modified nanopore at +100 mV.

Figure 4.

Detection of IgE in the external solution with an A_{IgE} -modified nanopore. **a.** A model showing the detection of protein presented in the exterior solution (left) and the expected current trace (right). **b.** Current in an 55 nm A_{IgE} -encoded nanopore at -100 mV with 5 nM IgE in the external solution. **c.** Nanopore current for addition of 5 nM IgE in the pipette used in panel a. **d.** The detection time interval between the first and the second IgE block ($t_1 - t_2$) in various IgE concentrations ($[IgE]$).

Figure 5. Single-molecule detection of ricin A-chain protein in the external solution using an A_{Ricin} -encoded nanopore. **a.** Current in a 56 nm A_{Ricin} -encoded nanopore at -100 mV, with 100 nM ricin A-chain protein in the external solution. **b.** The detection time interval between the first and the second ricin block ($t_1 - t_2$) in various concentrations of ricin A-chain protein.